Uptake and effects of 2, 4, 6 - trinitrotoluene (TNT) in juvenile Atlantic salmon (*Salmo salar*)

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ABSTRACT

Organ specific uptake and depuration, and biological effects in Atlantic salmon (*Salmo salar*) exposed to 2, 4, 6-trinitrotoluene (TNT) were studied. Two experiments were conducted, the first using radiolabeled TNT (^{14}C-TNT, 0.16 mg/L) to study uptake (48 h) and depuration (48 h), while the second experiment focused on physiological effects in fish exposed to increasing concentrations of unlabeled TNT (1 µg – 1 mg/L) for 48 h. The uptake of ^{14}C-TNT in the gills and most of the organs increased rapidly during the first 6 hours of exposure (12 hours in the brain) followed by a rapid decrease even though the fish was still exposed to TNT in the water. The radioactivity in the gall bladder reached a maximum after 55 hours, 7 hours after the transfer to the clean water. A high concentration of ^{14}C-TNT in the gall bladder, indicates that the TNT is excreted through the gall bladder. Mortality (2 out of 14) was observed at a concentration of 1 mg/L, and the surviving fish had hemorrhages in the dorsal muscle tissue near the spine. Analysis of the physiological parameters in blood from the high exposure group revealed severe effects, with an increase in the levels of glucose, urea and HCO_3, and a decrease in hematocrit and the levels of Cl and hemoglobin. No effects on blood physiology were observed in fish exposed to the lower concentrations of TNT (1-100 µg/L). TNT and the metabolites 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) were found in the muscle tissue, whereas only 2-ADNT and 4-ADNT were found in the bile. The rapid excretion and estimated bioconcentration factors (range of 2-18 after 48 h in gills, blood, liver, kidney, muscle and brain) indicated a low potential for bioaccumulation of TNT.

Key words: Trinitrotoluene; TNT; Atlantic salmon; dumped ammunition; bioaccumulation; toxicity; blood chemistry

1. Introduction

2, 4, 6-Trinitrotoluene (TNT), is a widely used military explosive. TNT has a low melting point, high stability, and low sensitivity to impact, friction and high temperature. Wastewater, soils, groundwater, and surface waters have become contaminated with a variety of energetic compounds, such as TNT, arising from ammunition manufacture and processing, training activity at firing ranges, as well as ammunition dumping (Spalding and Fulton, 1988; Levsen et al., 1993; Bradley et al., 1994; Ampleman et al., 2004; Rosen et al., 2016; Voie and Mariussen 2017). It has been estimated that between 750000 and 1.5 million metric tons of conventional ammunition was dumped along the German North Sea cost after the Second World War (Marencic and Nehring, 2009). This contamination is of concern and may pose a threat to exposed animals and plants. Fish appears to be the most sensitive species to TNT exposure. Lethal concentrations of TNT to fish are in the range of 0.8-5.0 mg/L water (Liu et al., 1983). Studies on fish have revealed that TNT affects haematological parameters, such as increased numbers of white blood cells and methaemoglobin (Ek et al., 2003). This is in accordance with effects on poisoned humans, of which TNT exposure in the early stages may cause a stimulation of the bone marrow resulting in an increase in the number of erythrocytes and leukocytes (Ryon and Ross, 1990). The intoxication may then be followed by a destruction of the hemoglobin in the cells, or by a chemical change in the hemoglobin of the red blood corpuscles, forming mixtures of methheamoglobin, NO-haemoglobin, and sulphhaemoglobin (Ryon and Ross, 1990), with a consequent damage of the oxygen-carrying functions. Sensini et al (2008) exposed European eel to TNT in the water and observed that TNT induced structural lesions on the gills, such as oedema and vascular congestion in addition to mucus hyper secretion. Behavior response of the fathead minnow, such as lethargy and

loss of motor control, has also been measured after exposure to TNT in 96 hours at a concentration of 0.46 mg/l (Smock et al., 1976).

TNT has a relatively high water solubility and several studies on different aquatic organisms, such as fish, mussels, tadpoles and amphipods have shown that TNT has a low potential for bioaccumulation (Lotufo and Lydy, 2005; Lotufo et al., 2016; Lotufo et al., 2015; Sims and Steevens, 2008). In addition, studies on aquatic organisms have shown a rapid metabolization of TNT when taken up, with an elimination half-life of less than an hour (Ownby et al., 2005; Yoo et al., 2006). These findings build up onto the fact that few studies have detected TNT or metabolites of TNT in aquatic organisms, and only in invertebrates and kelp close to a source (e.g. Barton and Porter, 2004; Rossland et al., 2010). Bearing in mind the high toxicity of TNT to fish it is reasons to believe that they may be affected when bomb shells breaks and explosive materials are released by dissolution to the surrounding sediments and water column. In close vicinity of dumped ammunition it have in a few studies been measured concentrations of TNT and breakdown products of TNT in the water which may be harmful for aquatic organisms (Barton and Porter 2004; Rodacy et al., 2001). Due to dilution effect a rapid drop in the concentrations of munitions residues in water is, however, expected and mobile organisms such as fish is probably only subjected to acute short-time exposure. The fast depuration rate of TNT in the organisms and the dilution effect may make it challenging to identify a TNT exposure by established methods.

Some studies have investigated organ distribution of TNT in fish. Ownby et al., (2005) showed that TNT was associated to the gills and internal viscera of channel catfish (*Ictalurus punctatus*) exposed to ^{14}C-TNT in the water. Ek et al., (2005) showed that considerable amount of TNT residues could be found in the fish bile from

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juvenile rainbow trout (*Oncorhynchus mykiss*) as conjugated metabolites after intraperitoneal exposure. The fish bile may therefore be a useful biomarker to identify TNT exposed fish at ammunition dump site. Ek et al., (2005) exposed the fish by intraperitoneal injection.

Most likely the fish is exposed to explosive residues dissolved in the water and to test the hypothesis that TNT accumulates in the fish bile we investigated the fate of TNT in water exposed juvenile Atlantic salmon (*Salmo salar*). The study was performed in two separate experiments: Atlantic Salomon were exposed to ^{14}C-labeled TNT dissolved in water to investigate uptake and elimination of ^{14}C-TNT in 8 different organs. The fish were exposed to ^{14}C-TNT for 48 hours followed by 48 hours in clean water to observe the elimination pattern. In light of the depuration experiment, Atlantic salmon were exposed to increasing concentrations of non-labeled TNT. The fish were subjected to analysis of blood physiology and analyzed for TNT and two of its major breakdown products, 2-aminodinitrotoluene (2-ADNT) and 4-aminodinitrotoluene (4-ADNT), in muscle tissue and bile. The measured concentrations of TNT and its breakdown products in the bile as a function of exposure concentration may give an indication of the usefulness of the bile as a biomarker for TNT-exposure.

2. Material and Methods

2.1. Chemicals

A stock solution was made of TNT dissolved in 75% ethanol and 25% acetonitrile. The TNT was a gift from Ms Tove Karsrud at FFI. Analysis of the stock solution showed small amounts of impurities (< 0.1%) such as 2-ADNT, 4-4A-DNT, 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 1,3,5-trinitrobenzene (TNB) and 1,4-dinitrobenzene (DNB). For the chemical analyses, the following unlabeled standards were diluted with acetonitrile (HPLC grade) to various concentrations: 2, 4, 6-trinitrotoluene (TNT) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). 2-ADNT and 4-ADNT were purchased from Ultra Scientific (Kingstone, RI). Isotope labelled 2, 4, 6-TNT ($^{13}C_7$) from Cambridge Isotope Laboratories were used as an internal standard. β-glucuronidase was purchased from Sigma-Aldrich (Norway). ^{14}C labeled TNT with a specific activity of 5 mCi/mmol and a concentration of 0.1 mCi/ml was purchased from American Radiolabeled Chemicals (St. Louis, MO).

2.2. Fish

Juvenile Atlantic salmon (*Salmo salar*) were bred at the Fish Laboratory of Norwegian University of Life Sciences (NMBU, Ås, Norway). Fish of both genders were approximately 6 months of age at the start of the experiments. Feeding of the fish ceased 5 days before the experiments started.

2.3. Fish exposure to ^{14}C-labeled TNT

Juvenile Atlantic salmon (n = 144) with a length and body weight of 14.1 ± 1.1 cm and 24.9 ± 5 g (mean ± SD) were split in two aquariums, one control tank and one

tank in which the fish were exposed to ^{14}C-TNT. The sub-optimal design with all fish in one aquarium was to limit possible contamination in the lab with the radiolabeled substance. The fish were kept in 90 L black polyethylene tanks with 50 L of water brought from Lake Maridalsvannet in Oslo (Mahrosh et al., 2014). The mean water temperature and oxygen content was 10°C and 9.7 mg/L and stable throughout the experiment. An increase in pH was observed during the experiment, from 6.7 at the start to 7.5 at the end. The fish were not fed during the experimental period. The exposure tank was added 1.8 ml ^{14}C labeled TNT with a specific activity of 5 mCi/mmol and a concentration of 0.1 mCi/ml corresponding to a concentration of 0.163 mg/L or 0.72 µM.

Fish were sampled (n=6) after 1, 3, 6, 12, 24 and 48 h of exposure. After 48 h the remaining fish were transferred to clean water to study the depuration of ^{14}C-TNT. During the depuration phase, six fish were sampled after 1, 3, 6, 12, 24 and 48 h respectively. The fish were sacrificed with a blow on the head and blood was immediately collected from the caudal vein with a heparinized syringe. The sampling procedure followed the EMERGE (Rosseland et al. 2001) and ICP Waters (ICP Waters 2010; http://www.icp-waters.no/publications/#icpwmanual) manuals. Body weight and fish length measures were obtained from individual fish. After the blood sampling, the fish were dissected and the gills, gall bladder, kidney, intestine, brain and muscle tissue were put into pre weighted scintillation vials. Each scintillation vial was weighed and added 1-2 ml Solvable® (Perkin Elmer, Waltham, USA) to dissolve the tissues and incubated at 60°C at a water bath for 60-150 minutes. With the exception of the brain and muscle samples, each vial was then added 200 µl 30 % H_2O_2 to improve the oxidation of the tissues and incubated for additional 30-60 minutes at 60°C in a water bath until a clear solution was obtained. Each vial was then

added 10-15 ml of Hionic Fluor® scintillation or Ultima Gold® (brain and muscle samples) and counted for retained radioactivity in a liquid scintillation spectrophotometer (Packard Tri-Carb 300). To estimate retained radioactivity in the water 5 ml of water sample was added 10 ml Ultima Gold in a scintillation vials and counted for retained radioactivity in a liquid scintillation spectrophotometer.

2.4. Fish exposure to increasing concentrations of unlabeled TNT

Juvenile Atlantic salmon (n = 49) with length and body weight of 14.7 ± 0.5 cm and 27.9 ± 2.6 g (mean ± SD) was distributed to 7 black polyethylene barrels in which it was put large polyethylene plastic bags. Each tank was filled 100 L of water and added 7 fish representing untreated control, solvent control, and 5 TNT exposed groups basically as described in protocols for fish acute studies (OECD 1992; US-EPA 1996). TNT (10 g/L) was prepared in a stock solution of 25% acetonitrile and 75 % ethanol (vol/vol). The stock solution was diluted further in acetonitrile and ethanol (1:3 vol/vol) giving a final solvent concentration of 0.01% in each tank. The fish were exposed to increasing concentrations of TNT (nominal concentrations of 1, 10, 100, and 1000 µg/L and measured concentrations of 1.0 µg/L, 12.6 µg/L, 108 µg/L, 1014 µg/L respectively) in synthetic Lake Maridalsvann water (1.75 mg/L Ca^{2+}, 1.51 mg/L Mg^{2+}, 3.28 mg/L Na^{+}, 0,26 mg/L K^{+}, 8.57 mg/L CO_3^{2-}, 10.17 mg/L SO_4^{-}, 0.24 mg/L Cl^{-}, pH 7). In addition, one group of fish was exposed to a nominal concentration of 1000 µg/L (measured concentration, 1026 µg/L) in water from the Lake Maridalsvannet, which contains some organic carbon (approximately 4.5 mg/L total organic carbon, Mahrosh et al., 2014). The fish were exposed for 48 h at 4-5°C in the dark in a static exposure system served with air pumps. The fish were not fed during the experimental period. Interaction between individual fish in the same tank on the

measured parameters was not studied and considered low and probably of little significance for the main conclusion in the study.

2.5. Sampling procedure of TNT exposed fish

After 48 h of exposure the fish were sacrificed with a blow on the head and blood was collected from the caudal vein with a heparinized syringe for measurement of blood physiology using an i-STAT portable analyzer (Abbot East Windsor, U.S.). The blood parameters that were analyzed were pH, HCO_3 (mmol/L), Na (mmol/L), K (mmol/L), Cl (mmol/L), urea (mg/dL), hematocrit (% packed cell volume (PCV)), and hemoglobin (g/dL). Glucose (mmol/L) was analyzed with a FreeStyle Lite blood glucose monitoring system (Abbot, Alameda, USA) with OneTouch®Ultra® blood glucose test strips (LifeScan Europe, Zug, Switzerland). The fish were then dissected to collect the gall bladder and muscle tissue according to manuals. The tissues were frozen on liquid nitrogen and stored at -80°C before further analysis.

2.6. Procedures for extraction of TNT

Water samples were collected in glass bottles (Duran) and preserved with $NaHSO_4$ (1.2 g/L, pH approximately 2) and stored cooled (4 °C) and dark, protected from exposure to UV-light. Water samples with a volume of 50-500 ml were added internal standard and extracted using Porapak RDX solid-phase extraction cartridges (Waters Corporation, MA). The columns were cleaned with acetonitrile followed by ultrapure deionized water before the water samples were added to the columns. Acetonitrile (~5ml) was then added to the columns to elute the explosives. The volumes of the extracts were reduced (~2ml) by a gentle blow of nitrogen and filtrated through 0.45 µm syringe filters (Millipore).

Fish muscle tissue, approximately 1.5-2.5 g (w.w), was homogenized in an Ultra Turrax homogenizer, mixed with Na_2SO_4 (~20 g) and added to Teflon container with internal standard, distilled water (8 ml) and acetonitrile (30 ml) for microwave assisted extraction. The samples were extracted at 100°C in 10 minutes. After digestion, the samples were allowed to cool down to room temperature into their vessels before transfer to 250 ml Teflon bottles, and centrifuged (1500 rpm) for 10 minutes. The acetonitrile extracts were evaporated to approximately 2 ml followed by clean up on ISOLUTE ENV+ columns (Biotage). The columns were cleaned and reconditioned with acetonitrile before adding the extracts which were rinsed through the column with an additional volume (~8 ml) of acetonitrile. The volumes of the extracts were reduced (~2ml) by a gentle blow of nitrogen and filtrated through 0.45 µm syringe filters (Millipore). The volumes were reduced additionally with nitrogen to approximately 1 ml before analyses.

Extraction of TNT and its residues from bile was based on the method described by Ek et al. (2005) with minor modifications. Bile (25µl) was mixed with water and 900 units of β-glukuronidase and incubated for approximately 24 hours at 37°C. After incubation the samples were added acetonitrile (950 µl) and internal standard (150µl) with some Na_2SO_4 to remove water. The samples were then sonicated for 10 min and subjected to further clean up on ISOLUTE ENV+ columns as described for muscle tissue.

2.7 Instrumental parameters for chemical analysis of TNT

Chemical analysis was performed by LC-MS/MS using an ACQUITY UPLC coupled with a Xevo TQ mass spectrometer from Waters. For separation of the compounds an ACQUITY UPLC® BEH Phenyl (1.7 µm, 2.1 x 100 mm) column was

used. The mobile phase consisted of water (solvent A) and methanol (solvent B), both containing 2 mM ammonium acetate. The analytes were eluted with a linear gradient of 30-90 % solvent B from 0 to 6 min, with a constant flow rate of 0.300 mL/min. TNT, and the TNT metabolites 2 ADNT and 4-ADNT were analyzed by multiple reaction monitoring (MRM) of the compounds respective parent and daughter molecules formed by electrospray ionization (ESI) in negative mode, in principle as described by Lotufo et al. (2016). Quantification was performed with 2, 4, 6-TNT ($^{13}C_7$) as internal standard. TNT was monitored at m/z 226 $[TNT-H]^-$ (parent ion) and m/z 197 $[TNT-H-NO]^-$ (daughter ion); the latter was used for quantification. 2-ADNT and 4-ADNT coeluted on the separation column, and had identical parent ions $[M-H]^-$ of m/z 196. However, the intensity of the daughter ions differed which enabled us to differentiate between the two compounds. The daughter ions selected for quantitation of 2-ADNT and 4-ADNT was m/z 136 $[M-H-2NO]^-$ and m/z 149 $[M-H-HNO_2]^-$, respectively. The fragmentation patterns of 2-ADNT and 4-ADNT revealed some presence of m/z 149 (~30%) and m/z 136 (~15%), respectively, implying that the quantified results are somewhat overestimated. The results of 2-ADNT and 4-ADNT must therefore be considered semi quantitative.

2.8. Data analysis.

Bioconcentration factors (BCF) in the depuration experiment were estimated as µg ^{14}C-TNT equivalents per kg tissue divided by µg ^{14}C-TNT equivalents per L of water at the same time point as the fish were sampled. BCF in the second experiment were estimated as µg TNT per kg tissue divided by µg TNT per L in the exposure waters at the end of the experiment. Elimination half-time was estimated from non-linear regression one-phase exponential decay. Descriptive statistics, non-linear

regression analysis (one phase exponential decay), ANOVA and mathematical calculations were computed in GraphPad Prism 5 or Excel 2007.

3. Results

3.1. TNT in the exposure water

The radioactivity in the water decreased during the first 10 h followed by a subsequent increase (Fig 1). The radioactivity in the water in the depuration tank increased during the first 20 h after the transfer of the fish (Fig 1).

In the second experiment, water was analyzed for TNT, 2-ADNT and 4-ADNT. Water from the respective exposure tanks were analyzed at the start of the experiment and after approximately 8 h, 24 h and 48 h showing a gradually a decrease of TNT in the water as a function of time. After 48 h the reduction in the TNT concentration ranged from 78% in the 100 µg/L tank to 16% in the water from the Lake Maridalsvannet (Table 1). The concentrations of 2-ADNT and 4-ADNT increased during the first 24 h followed by a decrease (Table 2), except in the water from the Lake Maridalen.

3.2. Uptake and excretion of TNT in fish

In the gills, blood, liver, kidney, muscle and the brain it was a rapid increase in the uptake of ^{14}C-TNT with a maximum tissue concentration after 6 hours (12 hours in the brains) (Fig 2). After 6 hours (12 hours in the brains) the concentration of ^{14}C-TNT in the organs started to decrease, even though the fish was still exposed to ^{14}C-TNT in the water. The radioactivity in the gallbladder increased during the whole experiment, reaching a maximum after 55 hours, 7 hours after the transfer to the clean water (Fig 3). A similar trend was observed in the intestines. The excretion of ^{14}C-TNT in the fish started before they were transferred to the clean water. Estimated elimination half-time of ^{14}C-TNT in the fish from the time the depuration started ranged from 8 hours in the liver to 22 hours in the kidney (Fig 1S, Supplementary materials). It was not feasible

to make a good estimation of the elimination half-life of ^{14}C-TNT in the fish after the transfer to the clean water since the concentration in the different organs already was near the measured minimum levels.

BCF were estimated after 6 hours of exposure when maximum tissue concentrations were observed and after 48 hours when the fish were transferred to the clean water. With the exception of the gallbladder the BFCs were highest in the gills, kidney, livers and intestine representing the first target of exposure and organs of excretion and metabolism (Table 3). The BCFs in these organs decreased considerable after 6 hours of exposure. The BCF of ^{14}C-TNT in the gall bladder increased from 404 after 6 hours of exposure to nearly 2000 after 48 hours of exposure.

In fish exposed to unlabeled TNT, TNT, 2-ADNT and 4-ADNT were found in the muscle tissue. Only 2-ADNT and 4-ADNT were found in the bile samples (Table 4 and 5). Bioconcentration factors were estimated based on the concentration in the tissue and the concentrations in the water and was between 4 and 6 (Table 4)

3.3 Effects of increasing TNT concentrations on Atlantic salmon

One fish in each of the two groups exposed to the highest concentration (1 mg/L) died during the experiment. During the dissection procedure it was observed that all the fish exposed to the highest concentrations had severed hemorrhages in the dorsal muscle tissue near the spine (Fig 2S). All the fish exposed to the highest concentrations would probably have died if the exposure time had been extended. There were no visible signs of any injuries or behavioral abnormalities on the fish exposed to the lower concentrations. With the exception of the groups exposed to the highest concentrations there were no significant effects on any of the blood physiology parameters that were analyzed. Severe effects on blood physiology was observed in

the two groups exposed to 1 mg/L of TNT (Table 6 and 7). In fish exposed to synthetic lake water, increased levels of glucose, urea and HCO_3 were observed, while hematocrit and the levels of Cl and hemoglobin (Table 6) decreased. In the fish exposed to TNT in the water from Lake Maridalsvannet it was observed an increase in urea and glucose, and a decrease in hematocrit and the levels of hemoglobin (Table 7).

4. Discussion

4.1. TNT in the exposure water

The experiment with use of ^{14}C-labeled TNT showed a loss of approximately 25% of the added ^{14}C-TNT from the water during the first 12 hours followed by a subsequent increase in the radioactivity (Fig 1). The subsequent increase in the radioactivity is probably attributed to excretion from the fish, bearing in mind that the depuration started before the fish were transferred to the clean water. At the end of the experiment a reduction in total radioactivity of 12% was observed. The loss may primarily be attributed to uptake and sorption in the fish, but may also be due to adsorption to the exposure tank walls made of polyethylene which has hydrophobic properties. In the second experiment with unlabeled TNT the findings were more complex. In all the exposure groups a reduction in the TNT concentrations were observed in the water, ranging from 60 to 80% reductions in the groups exposed to 1-100 µg/L and a 15-40% reduction in the groups exposed to 1 mg/L (Table 1). The loss of TNT during the experiment indicated uptake and/or metabolism in the fish, attachment to the tank walls, and/or decomposition in the water. Apparently the reduction in the TNT concentration was less pronounced in the water from the Lake Maridalsvannet, of which it was observed a loss of approximately 15%. Water from Lake Maridalsvannet was also used in the first experiment, which had a similar loss of radioactivity during the experiment. Unlike the synthetic water, the water from Lake Maridalsvannet contain organic materials (approximately 4.5 mg/L), which TNT and metabolites may be associated with. It has previously been shown that TNT may be adsorbed to organic materials in soil, such as humic acids (Esteve-Nunez et al., 2001; Eriksson et al., 2004). The metabolites of TNT, 2-ADNT and 4-ADNT, were detected in the water. The amount of metabolites, could however, not account for the loss of

TNT, indicating formation of considerable amount of unknown decomposition products, or adsorption to the tank walls and to the fish. In the ^{14}C-TNT experiment a considerable fraction of radioactivity retained in the different organs at the end of the study (Fig 2). This observation indicates that some of the losses of TNT in the water may be due to non-extractable TNT-compounds adsorbed to fish tissues. Previous studies on effects of TNT on aquatic organisms have shown similar losses of TNT in the exposure water (Conder et al., 2004; Lotufo et al., 2010; Yoo et al., 2006). Possible decomposition products other than the ADNT, may be diamino nitrotoluenes, tetranitroazoxytoluenes, nitrobenzoic acids and nitrogen free TNT transformation products (Conder et al., 2004; Preiss et al., 2005; Esteve-Nunez et al., 2001; Carpenter et al., 1978; Kaplan and Kaplan, 1982; Smith et al., 2015).

4.2. Uptake and excretion of ^{14}C-TNT in fish

A rapid uptake of ^{14}C-TNT was observed in the gills, blood, liver, kidney, muscle and the brain after 6 hours of exposure. In the brains maximum concentration was reached after 12 hours of exposure. The delayed uptake in brain demonstrate that the ^{14}C-TNT compounds passes the blood brain barrier. The maximum concentration of ^{14}C-TNT in the fish brain was similar to the concentrations detected in other tissues. After 6 hours in six of the tissues, and 12 hours in the brains, the concentration of ^{14}C-TNT started to decrease, even though the fish was still exposed in the water. Since the Atlantic salmon was not fed, and do not drink water, the site of TNT uptake must be the gills. The mechanism behind the reduced uptake after the initial rapid uptake is unclear, but it might be linked to negative effects on important processes at the gill surface and ion exchange mechanisms, demonstrated by the negative effects on osmoregulation and general physiology. Changes in mucus secretion or mucus quality

might also be an explanation, although not measured in our experiment. Sensini et al. (2008) exposed European eel to TNT in the water for concentrations ranging from 0.5 – 2.5 mg/L. In addition to lesions on the gills, such as oedema and vascular congestion, they observed mucus hypersecretion, which may be a response to an exposure to a xenobiotic leading to reduced uptake.

BCF were estimated after 6 hours of exposure to ^{14}C-TNT when maximum uptake was observed and after 48 hours when the exposure period was terminated. With the exception of the gall bladder the BCFs were highest in the gills, kidney, livers and intestine, representing first target of exposure and organs of excretion and metabolism (Table 3). The BCFs decreased considerable after 48 hours showing that ^{14}C-TNT is not particular prone to bioaccumulation and that it is readily excreted. The BCF of ^{14}C-TNT in the gall bladder increased from 404 L/kg after 6 hours of exposure to nearly 2000 L/kg after 48 hours of exposure. Apparently the ^{14}C-TNT was concentrated into the gall bladder showing that the fish primarily excrete TNT and potential metabolites through the bile. In most organs, the concentrations of ^{14}C-TNT were close to the measured minimum when the fish were transferred to clean water and elimination half-life could not be calculated. Previous studies have estimated elimination half-life in different fish species of an hour or less (Ownby et al., 2005, Lotufo and Lydy , 2005; Yoo et al., 2006). The elimination half-time of TNT in the fish was, however, estimated from the time the depuration started and ranged from 8 hours in the liver and 22 hours in the kidney (Fig 1S).

At the termination of the experiment there were still some residual ^{14}C-TNT left in the fish organs (Fig 2) indicating a slow elimination of a portion of the accumulated ^{14}C-TNT. This portion may be the so-called unextractable fraction, which may be biotransformation products covalently bound to tissue proteins and other

macromolecules as discussed by Ownby et al, (2005). The reductive metabolism of TNT to aminonitrotoluenes involves formation of several reactive metabolites, which have shown to form adducts with thiol groups (Leung et al., 1995).

4.3. Accumulation of TNT, 2-ADNT and 4-ADNT in the fish

TNT and its metabolites 2-ADNT and 4-ADNT, were detected in the muscle tissue (Table 4 and 5). The concentrations of TNT in the muscle tissue increased with the exposure concentrations. The calculated BCFs of TNT in muscle tissue were in the range of 4-6 L/Kg (Table 4) confirming that TNT is not particularly prone to bioaccumulation and that it is readily excreted. With the ^{14}C-TNT study in mind, however, it is reasons to believe that the accumulated concentrations of TNT in the effect study were higher earlier in the experiment, maybe by a factor of 3-5. The concentrations of the ADNT-metabolites were similar as the concentration of the parent molecule, indicating that a considerable fraction of the parent molecule is metabolized.

The ^{14}C-TNT experiment showed that the radio labeled TNT accumulated in the gall bladder. This was confirmed by chemical analysis of the bile of the TNT exposed fish. We did, however, only detect the presence of 2-ADNT and 4-ADNT and not the parent molecule (Table 5). The bile in our study was treated with β-glucuronidase during preparation. Previously Eek et al (2005) could not recover any TNT and TNT metabolites in the bile from fish orally exposed to TNT (100-400 mg/kg body weight). By treating the bile with β-glucuronidase in order to hydrolyze glucuronide conjugated TNT and TNT metabolites it was shown that considerable portion of the TNT was excreted as glucuronides. Eek et al., (2005) found that that the concentrations of the metabolites in the bile were a factor of approximately 10 higher

than the parent compound, which may explain the reason why we did not detect TNT in the bile in our study. In mammals, comparative studies have shown that the major excretion pathway of TNT is through urine primarily as the glucuronide conjugates (El-Hawari et al., 1981; Talmage et al., 1999). In fish it appears that a major portion of the TNT is metabolized followed by a phase II conjugation with glucuronides and biliary excretion.

In order to identify potential exposure of TNT near dumping sites of ammunition it may be reasonable to analyze the fish bile. In this study TNT metabolites were detected in bile from fish exposed to 10 µg/L. With improved analytical technics it will probably be possible to detect TNT and breakdown products in bile from fish exposed to lower concentrations (e.g. Ochsenberg et al., 2008). Due to dilution of munition residues in the seawater there are often no detection or only trace amounts of energetics residues that are detected near ammunition dump sites (e.g. Darrach et al., 1998; Ampleman et al., 2004; Ochsenberg et al., 2008; Rossland et al., 2010; University of Hawai, 2011). In close vicinity of the munitions, however, considerable concentrations have been reported. At the Bedford Basin, Halifax Novia Scotia, large amounts of ammunition from WWI and WWII are dumped (Rodacy et al., 2001). Water was sampled in close vicinity of the ammunition (≤ 1 m), and at one site it was found 14 µg/L TNT, 123 µg/L 4-ADNT and 108 µg/L 2-ADNT in the water. Barton and Porter (2004) performed a survey at the eastern end of Isla de Vieques, Puerto Rico which were used as a naval gunnery and bombing range between 1943 and 2003. In seawater inside a bomb it was found 66.4-105 mg/L TNT showing considerable dissolution of the energetic materials. Approximately one meter from the bomb the concentrations had dropped to 17.7 and 7.9 µg/L. In order to assess risk of exposure from leakage of very low concentrations of munitions residues from

dumping sites or training areas, other sampling strategies, such as passive samplers, may be more convenient (Rosen et al., 2016; Belden et al., 2015) or biomonitoring with transplanted blue mussels (Strehse et al., 2017).

4.4. Effects of TNT on fish

In the two groups exposed to the highest concentration mortality was observed, and severe hemorrhages in the muscle tissue at the dorsal fin near the spine (Fig 2S). The effect of TNT on fish is species dependent with reported acute lethal concentrations ranging from 1-5 mg/L, of which species like fathead minnow and channel catfish appear less sensitive than rainbow trout (Liu et al., 1983). A previous study reported a mean LC_{50}-concentration of 1.15 mg/L on rainbow trout exposed for 96 h under static condition (Liu et al., 1983). Compared with other aquatic organisms fish appears to be one of the most sensitive taxa to TNT intoxication (Liu et al., 1983; Ribeiro et al., 2012; Lotufo et al., 2013) and Atlantic salmon appears to be a particular sensitive species. Leffler et al., (2014) exposed alevins of Atlantic salmon to TNT wastewater for 40 days. In the high exposure group (2.1 mg/L TNT) they observed approximately 25% mortality after 14 days and 100% mortality after 40 days. In the group exposed to 0.41 mg/L they observed approximately 30% mortality after 40 days. In comparison to our experiment it is apparent that alevins are somewhat less susceptible than the juveniles.

The effect of TNT on mammals has been thoroughly studied and it is reasons to believe that similar mechanisms of toxicity are involved in the fish. One of the most pronounced symptoms of severe TNT intoxication in mammals is anemia (Crawford 1954; Dilley et al., 1982; Ryon and Ross, 1990; Bradley 2011). The symptoms are caused either by hemolysis of the red blood cells (as we saw in our experiment), by a

destruction of the hemoglobin in the cells, or by a chemical change in the hemoglobin of the red blood corpuscles, forming mixtures of methhemoglobin, NO-hemoglobin, and sulphhemoglobin with a consequent damage of the oxygen-carrying capacity. The analysis of the physiological parameters in blood confirmed severe effects in our experiment (Table 6 and 7). These effects on blood were only observed in the fish exposed to the highest concentration of TNT. Both the effect on morphology and on blood physiology appeared independent of the water quality. The fish exposed to TNT in the synthetic water had, however, effects on two more blood parameters, but it is reason to believe that similar effect eventually had happened also in the fish exposed to the TNT in the water from Lake Maridalsvannet. Eek et al., (2003) who exposed rainbow trout perorally to TNT (100 mg/kg - 400 mg/kg) for 48 h did not report any morphological changes but observed increased levels of methhaemoglobin and increased numbers of lymphocytes and leukocytes, which is in accordance with previous studies on mammals and the present study. The effect observed on the fish is probably due to formation of reactive metabolites of TNT. In mammals the nitro groups on the TNT undergo a reductive metabolism into aminonitrotoluenes. The reduction of TNT is a stepwise reaction with the conversion of TNT to the intermediates nitrosodinitrotoluene and hydroxylaminodinitrotoluene. The conversion of hydroxylaminodinitrotoluene to aminodinitrotoluene is a rate limiting step which may cause an accumulation of the substance. Nitrosodinitrotoluene and hydroxylaminodinitrotoluene are reactive metabolites which may covalently bind to macromolecules in the cells (Levine et al., 1990; Michels et al., 1994; Leung et al., 1995; Bakhtiar and Leung, 1997; Esteve-Nunez, et al., 2001). Apparently, in the high exposure group in our experiment a level of metabolites was reached that was detrimental for the fish.

4.5. Conclusions

The present study have shown that TNT is taken up primarily over the gills and rapidly excreted from fish via the bile. After 6 hours (12 hours in the brains) the concentration of TNT in the organs started to decrease, even though the fish was still exposed to TNT in the water. The depuration experiment showed that the BCF for TNT-equivalents were substantially higher in the gall bladder, likely due to presence in the bile, compared to the other organs. In order to identify potential exposure of TNT near dumping sites of ammunition it may be reasonable to analyze the fish bile. Mortality was observed amongst fish exposed to 1 mg/L TNT. Considerable amount of the TNT metabolites 2-ADNT and 4-ADNT, were detected in the muscle tissue of the fish, and severe hemorrhages in the dorsal muscle tissue near the spine was observed. Effects on blood parameters such as of glucose, urea, hematocrit and hemoglobin were observed in the fish exposed to the highest concentration (1 mg/L). Large amount of ammunition have been dumped in the oceans of the world during the last century, but due to dilution of munitions residues in the seawater there is less likely that fish are particular vulnerable to exposure. Benthic organisms are probably at highest risk of exposure (Pascoe et al., 2010; Voie and Mariussen, 2017). However, in an area with little exchange of water, like a small lake or a threshold fjord, or in close vicinity of the ammunition, dissolved TNT and other ammunition residues may reach toxic levels.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Table 1

Concentrations (µg/L) of TNT in the water as a function of time in the effect study with unlabeled TNT. The concentrations are based on chemical analyses of one sample collected at each time point. The numbers in brackets show portion of TNT in percent relative to the start concentrations. M = water from Lake Maridalsvannet.

Exposure groups	**Measured concentrations, µg/L (% of start conc.)**			
(µg/L)	**Start**	**8h**	**24h**	**48h**
1	1.0	1.1 (108)	0.4 (42)	0.3 (31)
10	12.6	11 (87)	7.14 (57)	4.8 (38)
100	108	101 (94)	58 (54)	24 (22)
1000	1014	990 (98)	780 (77)	567 (56)
1000 (M)	1026	939 (92)	927 (90)	864 (84)

Table 2

Concentrations (µg/L) of the TNT metabolites, 2-ADNT and 4-ADNT, in the exposure water as a function of time in the effect study with unlabeled TNT. The concentrations are based on chemical analyses of one sample collected at each time point. M = water from Lake Maridalsvannet.

Exposure	**2-ADNT (µg/L)**				**4-ADNT (µg/L)**			
groups (µg/L)	**Start**	**8h**	**24h**	**48h**	**Start**	**8h**	**24h**	**48h**
1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
10	< 0.1	0.2	0.4	0.3	< 0.1	0.4	1.1	1.4
100	0.35	1.5	2.4	1.8	0.5	2.9	8	7.4
1000	2.3	5.9	10	1.8	1.5	7.4	26	16
1000 (M)	< 0.1	0.4	12	17	0.4	1.0	9.8	16

Table 3

Estimated bioconcentration factors (BCF) (mean BCF (L/kg) ± SD, n = 6) of radiolabeled ^{14}C-TNT in the different organs after 6 h and 48 h of exposure.

Organ	**BCF (6 hours)**	**BCF (48 hours)**
Gills	46 ± 5.0	9.6 ± 0.5
Blood	10 ± 1.4	2.5 ± 0.6
Liver	43 ± 9.2	18 ± 3.0
Kidney	57 ± 9.4	13 ± 1.3
Muscle	14 ± 0.7	2.4 ± 0.4
Brain	15 ± 1.0	6.7 ± 0.5
Intestine	41 ± 3.9	94 ± 18
Gall bladder	404 ± 75	1933 ± 354

Table 4

The concentrations of TNT and TNT metabolites in muscle tissue, and estimated BCF of TNT. The results are shown as mean concentrations (mg/kg) ± SD from 4 fish.

Exposure (µg/L)	**TNT** (mg/kg)	**BCF TNT** (L/kg)	**2-ADNT** (mg/kg)	**4-ADNT** (mg/kg)
1	< 0.05	n.e.[a]	< 0.05	< 0.05
10	< 0.05	n.e.	< 0.05	< 0.05
100	0.15 ± 0.01	6.3 ± 0.6	0.04 ± 0.01	0.08 ± 0.05
1000	2.9 ± 0.6	5.2 ± 1.0	0.97 ± 0.08	1.1 ± 0.5
1000 (M)	3.5 ± 0.8	4.1 ± 0.9	1.2 ± 0.03	1.1 ± 0.4

[a] n.e = not estimated

Table 5

The analyzed concentrations of TNT and TNT metabolites in the bile. The results are mean concentrations (mg/L ± SD) in bile from 4 fish. The medians are shown in brackets. M= water from Lake Maridalsvannet

Exposure (µg/L)	**TNT (mg/L)**	**2-ADNT (mg/L)**	**4-ADNT (mg/L)**
1	< 2	< 2	< 2
10	< 2	1.1 ± 0.02 (0.7)	5.5 ± 3.8 (4.5)
100	< 2	12 ± 12 (6.8)	40 ± 26 (28)
1000[a]	< 2	29 ± 20 (36)	50 ± 33 (67)
1000 (M)	< 2	60 ± 42 (55)	56 ± 37 (53)

[a] Results from 3 fish

Table 6

Effects on blood physiology parameters of the fish exposed to TNT. The results are presented as mean concentration ± SD. The medians are shown in the brackets. Each group was compared to the control group (Ctr) by one-way ANOVA. * represent a significance level of $P < 0.05$, ***represent a significance level of $P < 0.001$.

Exposure (µg/L)	**HCO3 (mmol/L)**	**Na (mmol/L)**	**K (mmol/L)**	**Cl (mmol/L)**	***n***
0 (Ctr)	3.4 ± 0.8 (3.2)	147 ± 2.9 (147)	2.9 ± 1.2 (2.3)	134 ± 1.1 (134)	5
0 (Solvent)	3.7 ± 0.7 (3.5)	145 ± 2.6 (146)	4.8 ± 1.2 (4.9)	133 ± 3.0 (134)	6
1	3.5 ± 0.9 (3.2)	145 ± 3.4 (146)	5.2 ± 1.1 (4.9)	136 ± 1.1 (136)	6
10	3.8 ± 1.0 (3.9)	144 ± 2.0 (145)	5.3 ± 1.0 (5.3)	135 ± 3.1 (135)	6
100	2.9 ± 0.4 (3.1)	141 ± 2.8 (141)	4.9 ± 0.7 (5.0)	134 ± 1.4 (134)	6
1000	9.5 ± 3.8 (8.3)***	137 ± 2.1 (138)	4.3 ± 0.4 (4.3)	115 ± 2.1 (115)***	6
1000 (M)	3.0 ± 1.0 (2.5)	141 ± 4.3 (139)	5.9 ± 1.4 (5.5)	137 ± 2.8 (138)	5

Table 7

Effects on blood physiology parameters of the fish exposed to TNT. The results are presented as mean concentrations ± SD. The medians are shown in the brackets. Each group was compared with the control group (Ctr) by one-way ANOVA. * represent a significance level of $P < 0.05$, ***represent a significance level of $P < 0.001$.

Exposure (µg/L)	**Glucose (mmol/L)**	**Urea (mg/dL)**	**Hct (% PCV)**	**Hb (g/dL)**	***n***
0 (Ctr)	3.7 ± 0.8 (3.9)	< 1	42 ± 3.4 (42)	14.1 ± 1.1 (14.3)	5
0 (Solvent)	4.7 ± 1.3 (4.8)	< 1	36 ± 3.3 (37)	12.1 ± 1.1 (12.6)	6
1	4.4 ± 1.1 (4.6)	< 1	37 ± 3.0 (37)	12.4 ± 1.0 (12.4)	6
10	3.5 ± 0.8 (3.5)	< 1	35 ± 5.6 (35)*	11.8 ± 1.9 (11.9)	6
100	3.7 ± 0.3 (3.8)	< 1	37 ± 2.7 (37)	12.4 ± 0.9 (12.6)	6
1000	14 ± 2.7 (13)***	1.5 ± 0.5 (1.3)***	16 ± 3.3 (15)***	5.4 ± 1.1 (5.1)***	6
1000 (M)	11 ± 3.0 (12)***	2.6 ± 0.5 (2.8)***	20 ± 2.6 (19)***	6.8 ± 0.9 (6.5)***	5

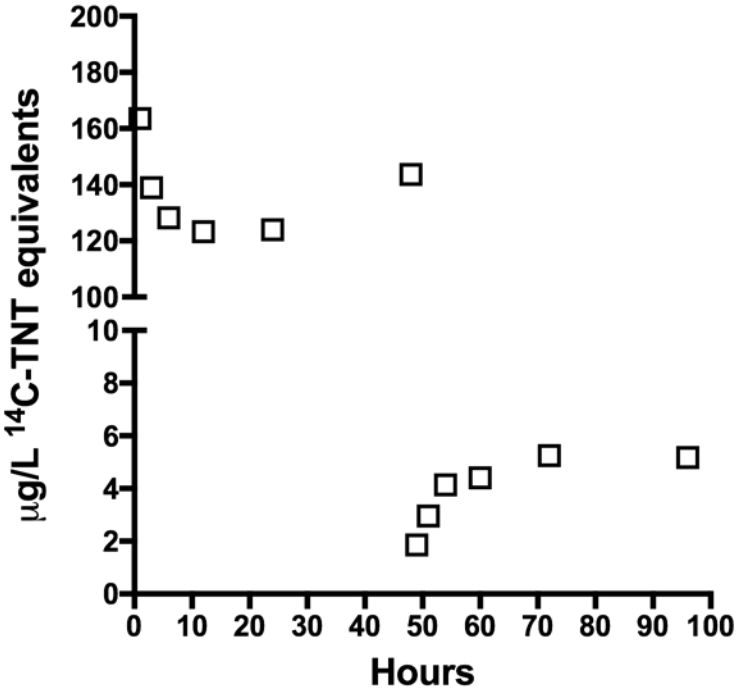

Fig 1. Measured radioactivity in the water shown as µg/L ^{14}C-TNT equivalents. After 48 hours the fish were transferred to clean water showing an increase in radioactivity, probably due to excretion from the fish.

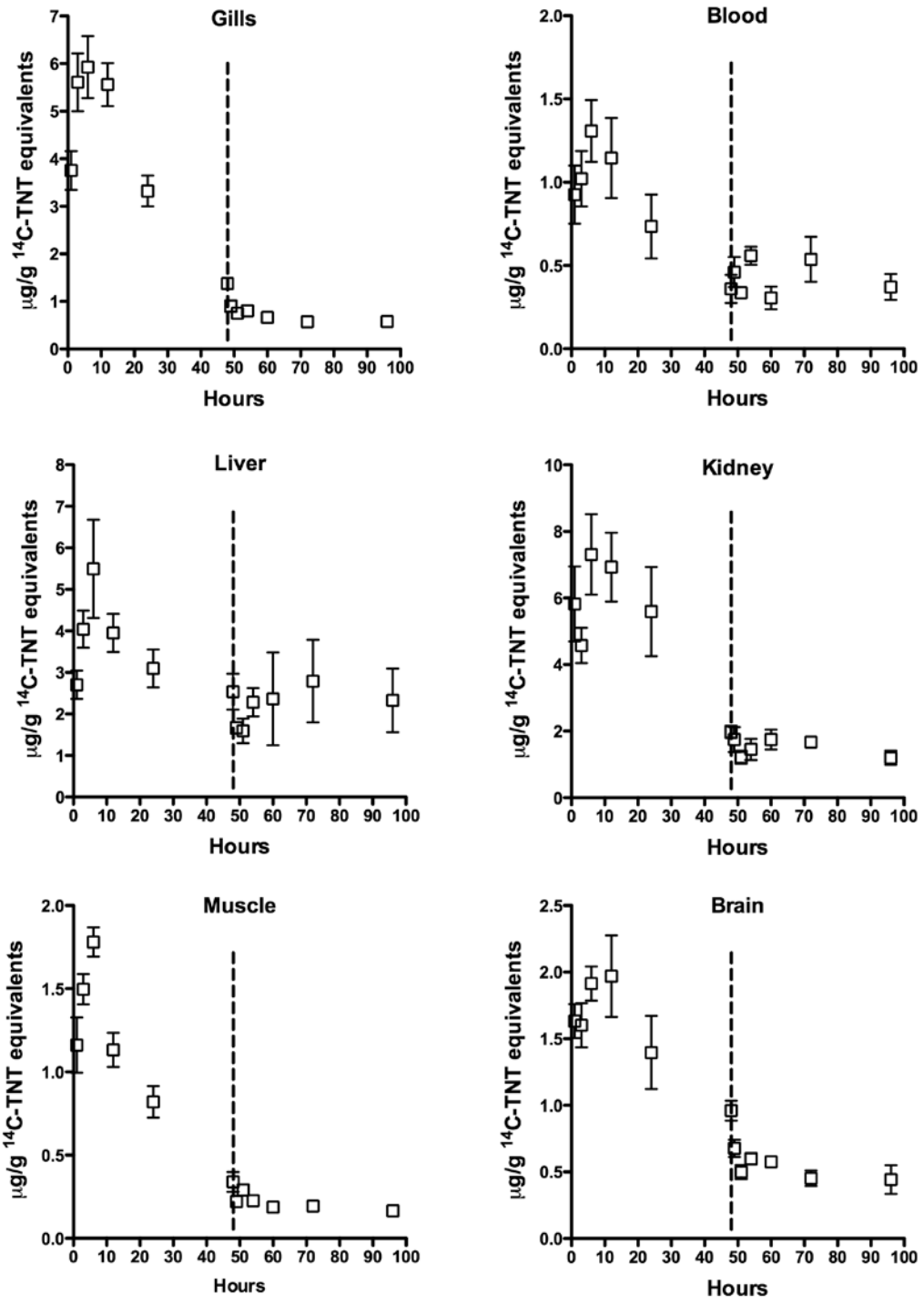

Fig 2. Uptake and excretion of ^{14}C-TNT as a function of time in different organs. After 48 h the fish were transferred to clean water showing depuration of the ^{14}C-TNT. Each data point represent mean (± SD) from 6 fish.

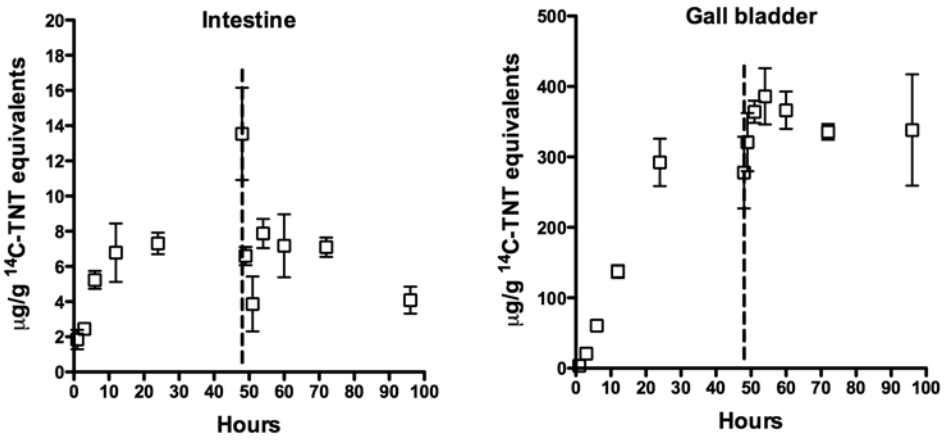

Fig 3. Uptake and excretion of ^{14}C-TNT as a function of time in the intestine and gall bladder. After 48h the fish were transferred to clean water showing depuration of the ^{14}C-TNT. Each data point represent mean (± SD) from 6 fish.

Supplementary materials

Uptake and effects of 2, 4, 6 - trinitrotoluene (TNT) in juvenile Atlantic salmon (*Salmo salar*)

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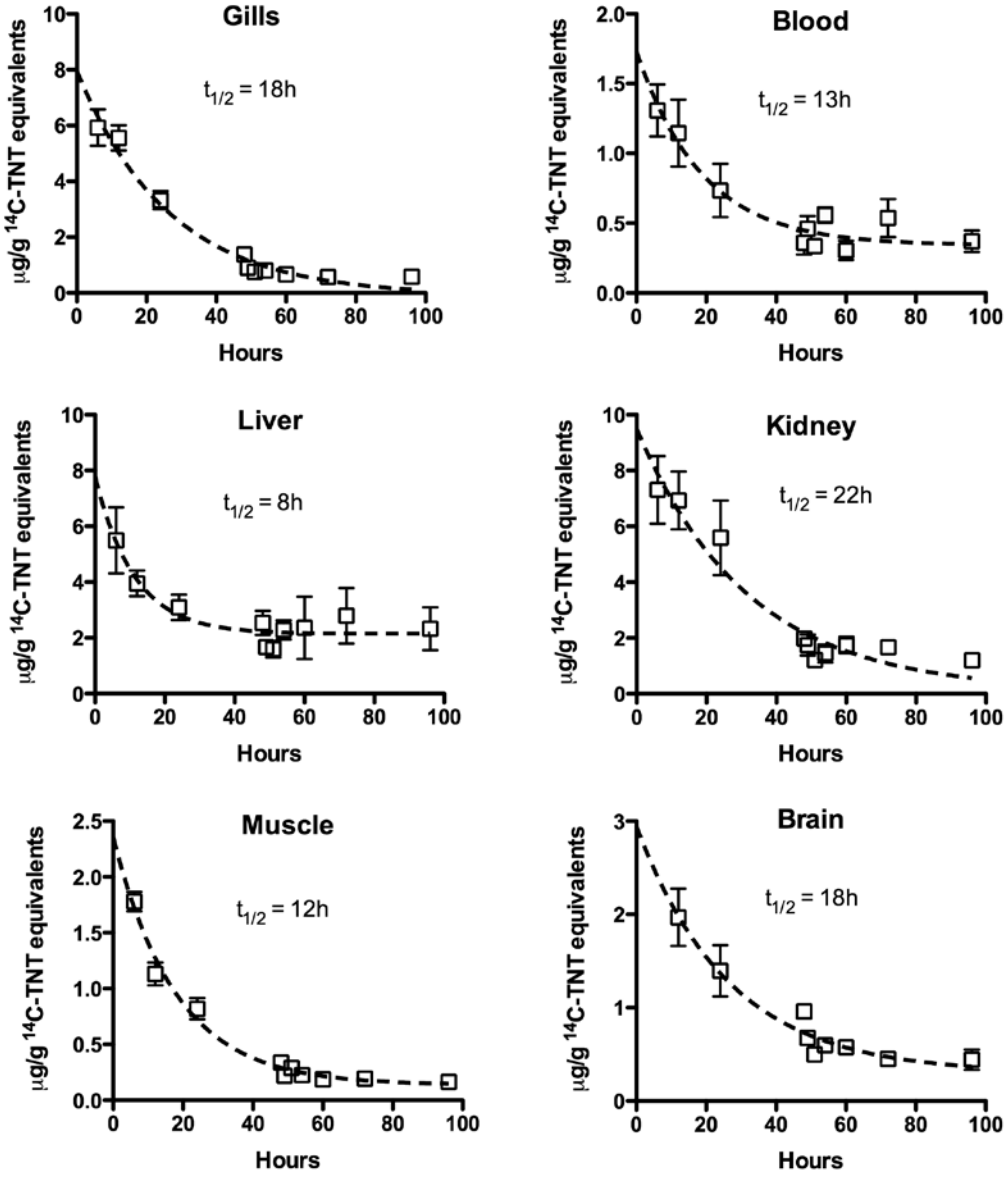

Fig 1S. Estimated elimination half-time of ^{14}C-TNT in different organs collected from the fish from the time the depuration started. Each data point represent the mean from six fish ± SD. Elimination half-time is estimated from non-linear regression one-phase decay. The results after 48 h represents retained radioactivity in the fish after transfer to clean water

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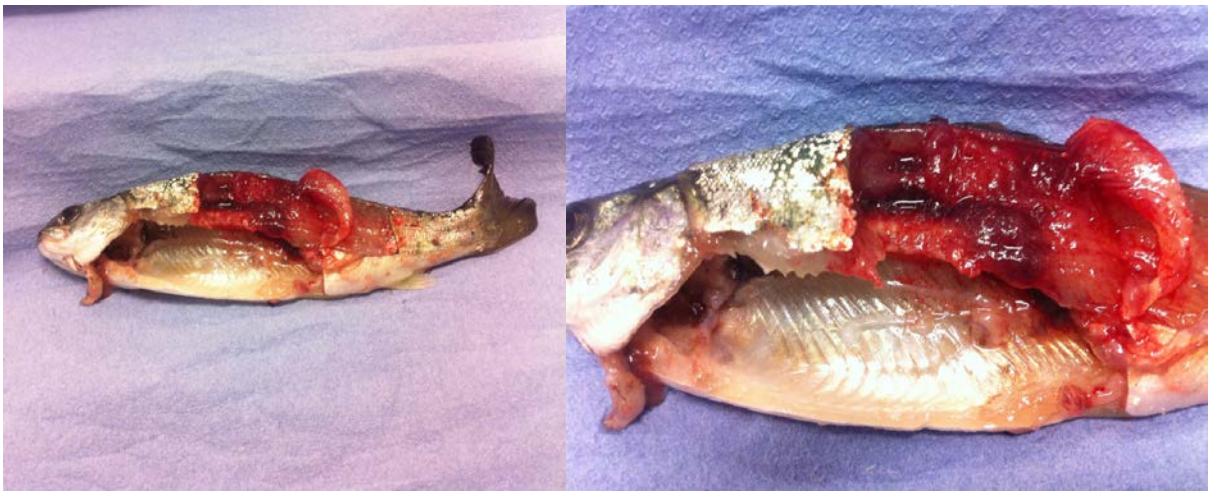

Fig 2S. Juvenile Salmon exposed to 1 mg/L TNT with hemorrhages in the dorsal muscle tissue near the spine.

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